

FORM PTO-1390 (REV. 10-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 20522P
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/089787
INTERNATIONAL APPLICATION NO. PCT/US00/27056 ✓	INTERNATIONAL FILING DATE September 29, 2000 ✓	PRIORITY DATE CLAIMED October 4, 1999 ✓
TITLE OF INVENTION MraY GENE AND ENZYME OF PSEUDOMONAS AERUGINOSA		
APPLICANT(S) FOR DO/EO/US Mohamed El-Sherbeini, Barbara A. Azzolina		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is an express request to begin national examination procedures [35 U.S.C. 371(f)] at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l). <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made and the US was elected by the expiration of the 19th month from the earliest claimed priority date (PCT Article 31). <input checked="" type="checkbox"/> A copy of the International Application as filed [35 U.S.C. 371(c)(2)]. a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <input type="checkbox"/> An English language translation of the International Application as filed [35 U.S.C. 371(c)(2)]. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C. 371(c)(3)]. a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)]. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) [35 U.S.C. 371(c)(4)]. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C. 371(c)(5)]. 		
Items 11 to 16 below concern other document(s) or information included:		
<ol style="list-style-type: none"> <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. <input type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. <input type="checkbox"/> A substitute specification. <input type="checkbox"/> A change of power of attorney and/or address letter. <input type="checkbox"/> Other items or information: 		

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U.S. APPLICATION NO (If known, see 37 CFR 1.5) 10/089787	INTERNATIONAL APPLICATION NO PCT/US00/27056	ATTORNEY'S DOCKET NUMBER 20522P			
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE [37 CFR 1.492(a)(1)-(5)]: Neither international preliminary examination fee (37 CFR 1.482) nor international search fee [37 CFR 1.445(a)(2)] paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee [37 CFR 1.445(a)(2)] paid to USPTO \$740.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =		CALCULATIONS			
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date [37 CFR 1.492(e)].		\$710.00			
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Claims	Number Filed	Number Extra	Rate		
Total Claims	16 - 20 =	0	X \$18.00	\$0.00	
Independent Claims	5 - 3 =	2	X \$84.00	\$168.00	
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =		\$878.00			
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.					
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Fee for recording the enclosed assignment [37 CFR 1.21(h)]. The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.		+ \$0.00			
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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive [37 CFR 1.137(a) or (b)] must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
<p>MERCK & CO., INC. Patent Department, RY60-30 P.O. Box 2000 126 East Lincoln Avenue Rahway, New Jersey 07065-0970</p>  <p>SIGNATURE</p>					
<p>DATE: <u>April 3, 2002</u></p> <p>PHONE #: <u>(732) 594-1273</u></p> <p>NAME <u>Anna L. Cocuzzo</u></p> <p>REGISTRATION NUMBER <u>42,452</u></p>					

DT05 Rec'd PCT/PTO 05 AUG 2002

10/089787

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	El-Sherbeini, Mohamed <i>et al.</i>	Authorized Officer:
Serial No.:	10/089,787	Case No.: 20522P
Filed:	April 3, 2002	
For:	MraY GENE AND ENZYME PSEUDOMONAS AERUGINOSA	

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

RESPONSE TO COMPLY WITH SEQUENCE REQUIREMENT

Sir:

The following statement is made as required by 37 C.F.R. § 1.821(f).

I hereby state that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821 (c) and (e), respectively, are the same and contain no new matter.

Respectfully submitted,

By 
Anna L. Cocuzzo
Reg. No. 42,452
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Date: August 5, 2002

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DATE OF DEPOSIT August 5, 2002
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WO 01/25251

TITLE OF THE INVENTION

MraY GENE AND ENZYME OF *PSEUDOMONAS AERUGINOSA*

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not Applicable

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not Applicable

10 REFERENCE TO MICROFICHE APPENDIX

Not Applicable

FIELD OF THE INVENTION

15 This invention relates to the genes and enzymes involved in cell wall synthesis in bacteria.

BACKGROUND OF THE INVENTION

The pathway of peptidoglycan (PG) biosynthesis is both essential and unique to bacteria and the responsible enzymes are present in both Gram-negative and 20 Gram-positive bacteria. Thus, inhibitors of these enzymes are likely to be broad spectrum and safe antibiotics. In fact, several enzymes in this pathway are molecular targets of naturally occurring antibiotics such as fosfomycin, cycloserine, β -lactams and vancomycin (Bugg & Walsh, 192 *Nat. Prod. Rep.* 9:199-215).25 One enzyme intrinsic to the peptidoglycan biosynthesis is MraY. To date, no MraY sequences have been disclosed for *Pseudomonas aeruginosa*, an opportunistic pathogen causing infections in patients with burns or neutropenia. More serious is its involvement in respiratory tracts of cystic fibrosis patients.It would be desirable to have polynucleotides and polypeptides 30 encoding the MraY protein of *Pseudomonas aeruginosa* in order to further screen compounds for antibiotic activity against this enzyme catalytically active in the first step of the membrane cycle of peptidoglycan biosynthesis. Inhibitors of this enzyme would be particularly helpful in preventing the growth of *Pseudomonads* and other G+C rich bacteria.

Possession of this information would also greatly facilitate determinations as to the role of the encoded enzyme, Phospho-*N*-Acetylmuramoyl-Pentapeptide-Translocase, in the pathogenesis of infection and disease.

5 SUMMARY OF THE INVENTION

Polynucleotides and polypeptides of *Pseudomonas aeruginosa* MraY, an enzyme involved in bacterial cell wall biosynthesis, are provided. The recombinant MraY enzyme is catalytically active in the first step of the membrane cycle of peptidoglycan biosynthesis, transferring the *N*-acetylmuramic acid pentapeptide to a bactoprenol phosphate carrier molecule. The enzyme is useful in *in vitro* assays to screen for antibacterial compounds that target cell wall biosynthesis. The invention includes the polynucleotides, proteins encoded by the polynucleotides, and host cells expressing the recombinant enzyme, probes and primers, and the use of these molecules in assays.

15 An aspect of this invention is an isolated polynucleotide having a sequence encoding a *Pseudomonas aeruginosa* MraY protein, or a complementary sequence. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In preferred embodiments the polynucleotide can be DNA, RNA or a mixture of both, and can be single or double stranded. In a most 20 preferred embodiment, the polynucleotide has a sequence shown in SEQ ID NO:1.

An aspect of this invention is a probe having a sequence of at least about 25 contiguous nucleotides that is specific for a naturally occurring polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. Probes in accordance with this description are useful for the specific detection of the presence 25 of a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. In preferred embodiments, the probes of this aspect can have additional components including, but not limited to, compounds, isotopes, proteins or sequences for ready detection.

An aspect of this invention is a primer having a sequence of at least about 25 contiguous nucleotides that is specific for a naturally occurring 30 polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. Primers in accordance with this description are useful in nucleic acid amplification-based assays for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. In preferred embodiments, the primers of this aspect can have additional components including, but not limited to, compounds, isotopes, proteins or sequences for ready detection.

- An aspect of this invention is an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In particular
- 5 embodiments, the vector can have any of a variety of regulatory regions known and used in the art as appropriate for the types of host cells the vector can be used in. In a most preferred embodiment, the vector has regulatory regions appropriate for the expression of the encoded protein in gram-negative prokaryotic host cells. In other embodiments, the vector has regulatory regions appropriate for expression of the
- 10 encoded protein in gram-positive host cells, yeasts, cyanobacteria or actinomycetes. In some preferred embodiments the regulatory regions provide for inducible expression while in other preferred embodiments the regulatory regions provide for constitutive expression. Finally, according to this aspect, the expression vector can be derived from a plasmid, phage, virus or a combination thereof.
- 15 An aspect of this invention is a host cell comprising an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In preferred embodiments, the host cell is a yeast, gram-positive bacterium,
- 20 cyanobacterium or actinomycete. In a most preferred embodiment, the host cell is a gram-negative bacterium.
- An aspect of this invention is a process for expressing a MraY protein of *P. aeruginosa* in a host cell. In this aspect a host cell is transformed or transfected with an expression vector including a polynucleotide encoding a *Pseudomonas*
- 25 *aeruginosa* MraY protein, or a complementary sequence. According to this aspect, the host cell is cultured under conditions conducive to the expression of the encoded MraY protein. In particular embodiments the expression is inducible or constitutive. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2.
- 30 An aspect of this invention is a purified polypeptide having an amino acid sequence of SEQ ID NO:2. Cellular extracts comprising a polypeptide having the above amino acid sequence are also included within the instant invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Nucleotide sequence (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO:2) of *P. aeruginosa* MraY. The amino acid sequence (SEQ ID NO:2) is presented in a three-letter code below the nucleotide sequence 5 (nucleotides 34 to 1113 of SEQ ID NO: 1).

DETAILED DESCRIPTION OF THE INVENTION

This invention provides polynucleotides and polypeptides of a cell wall biosynthesis gene from *Pseudomonas aeruginosa*, referred to herein as MraY.

10 The polynucleotides and polypeptides are used to further provide expression vectors, host cells comprising the vectors, probes and primers, and assays for the presence or expression of MraY.

15 Bacterial *mraY* encodes for phospho-*N*-acetylmuramoyl-pentapeptide translocase, an enzyme responsible for catalyzing the first step of the membrane cycle of peptidoglycan biosynthesis, transfer of the *N*-acetylmuramic acid pentapeptide to a bactoprenol phosphate carrier molecule.

The *mraY* gene was cloned from *Pseudomonas aeruginosa*. Sequence analysis of the *P. aeruginosa mraY* gene revealed an open reading frame of 361 amino acids.

20 Nucleic acids encoding MraY from *Pseudomonas aeruginosa* are useful in the expression and production of the *P. aeruginosa* MraY protein. The nucleic acids are also useful in providing probes for detecting the presence of *P. aeruginosa* MraY.

As used herein, the following definitions apply:

25 The term "about" in the specification means within approximately 10-20% greater or lesser than particularly stated.

30 The term "polynucleotide" means a nucleic acid of more than one nucleotide. A polynucleotide can be made up of multiple polynucleotide units that are referred to by description of the unit. For example, a polynucleotide can comprise within its bounds a polynucleotide(s) having a coding sequence(s), a polynucleotide(s) that is a regulatory region(s) and/or other polynucleotide units commonly used in the art.

35 The term "expression vector" means a polynucleotide having regulatory regions operably linked to a coding region such that, when in a host cell, the regulatory regions can direct the expression of the coding sequence. The use of

expression vectors is well known in the art. Expression vectors can be used in a variety of host cells and, therefore, the regulatory regions are preferably chosen as appropriate for the particular host cell.

The term "regulatory region" means a polynucleotide that can promote
5 or enhance the initiation or termination of transcription or translation of a coding sequence. A regulatory region includes a sequence that is recognized by the RNA polymerase, ribosome, or associated transcription or translation initiation or termination factors of a host cell. Regulatory regions that direct the initiation of transcription or translation can direct constitutive or inducible expression of a coding
10 sequence.

The terms "purified" and "isolated" are utilized interchangeably to stand for the proposition that the polynucleotide, protein and polypeptide, or respective fragments thereof in question have been removed from the *in vivo* environment so that they exist in a form or purity not found in nature. This, however,
15 is not mandated of cDNA as understood by one of ordinary skill in the art.

The term "substantially pure" with regard to a polynucleotide means it is obtained purified from cellular components by standard methods at a concentration of at least about 100-fold higher than that found in nature. A polynucleotide is considered essentially pure if it is obtained at a concentration of at least about 1000-fold higher than that found in nature.
20

Polynucleotides

Polynucleotides useful in the present invention include those described herein and those that one of skill in the art will be able to derive therefrom following
25 the teachings of this specification.

An aspect of the present invention is a polynucleotide encoding a MraY protein of *Pseudomonas aeruginosa*. It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that encode RNA
30 comprising alternative codons which code for the eventual translation of the identical amino acid. The present invention, thus, discloses codon redundancy which can result in different DNA molecules encoding an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation.

A further aspect of the present invention is a cDNA encoding a MraY protein of *Pseudomonas aeruginosa*.

5 A preferred aspect of the present invention is an isolated nucleic acid encoding a MraY protein of *Pseudomonas aeruginosa*. A preferred embodiment is a nucleic acid having the sequence disclosed in FIG. 1, SEQ ID NO:1.

The isolated nucleic acid molecule of the present invention can include a ribonucleic or deoxyribonucleic acid molecule, which can be single (coding or noncoding strand) or double stranded, as well as synthetic nucleic acid, such as a synthesized, single stranded polynucleotide.

10 Noncoding or antisense strands can be useful as modulators of the gene by interacting with RNA encoding the MraY protein. Antisense strands are preferably less than full length strands having sequences unique or specific for RNA encoding the polypeptide.

15 Also included in the present invention are polynucleotides that hybridize to *P.aeruginosa mraY* sequences under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C
20 in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

25 Other procedures using conditions of high stringency would include either a hybridization step carried out in 5 X SSC, 5 X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

30 Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, e.g., Sambrook, *et al.*, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Polypeptides

A preferred aspect of the present invention is a substantially purified form of the MraY protein from *Pseudomonas aeruginosa*. A preferred embodiment is a protein that has the amino acid sequence which is shown in FIG. 1, in SEQ ID NO:2.

Probes and Primers

Probes comprising full length or partial sequences of SEQ ID NO: 1 can be used to determine whether a cell or sample contains *P. aeruginosa mraY* DNA or RNA. A preferred probe is a single stranded antisense probe having at least the full length of the coding sequence of MraY. It is also preferred to use probes that have less than the full length sequence, and contain sequences specific for *P. aeruginosa mraY* DNA or RNA. The identification of a sequence(s) for use as a specific probe is well known in the art and involves choosing a sequence(s) that is unique to the target sequence, or is specific thereto. It is preferred that probes have at least about 25 nucleotides, more preferably about 30 to 35 nucleotides. The longer probes are believed to be more specific for *P. aeruginosa mraY* gene(s) and RNAs and can be used under more stringent hybridization conditions. Longer probes can be used but can be more difficult to prepare synthetically, or can result in lower yields from synthesis.

Examples of sequences that are useful as probes or primers for *P. aeruginosa mraY* gene(s) are Primer A (sense) 5'- TT CAT ATG CTC CTG CTG CTG GCC GAA TAC -3' (SEQ ID NO:3) and Primer B (antisense) 5'- TT GGA TCC TCA ACG CAG CTT CAA GGT G -3' (SEQ ID NO:4). Restriction sites, underlined, for NdeI and BamHI are added to the 5' ends of the primers to allow cloning between the NdeI and BamHI sites of the expression vector pET-11a. However, one skilled in the art will recognize that these are only a few of the useful probe or primer sequences that can be derived from SEQ ID NO:1.

Polynucleotides having sequences that are unique or specific for *P. aeruginosa* MraY can be used as primers in amplification reaction assays. These assays can be used in tissue typing as described herein. Additionally, amplification reactions employing primers derived from *P. aeruginosa* MraY sequences can be used to obtain amplified *P. aeruginosa mraY* DNA using the *mraY* DNA of the cells as an initial template. Many types of amplification reactions are known in the art and include, without limitation, Polymerase Chain Reaction, Reverse Transcriptase

Polymerase Chain Reaction, Strand Displacement Amplification and Self-Sustained Sequence Reaction. Any of these or like reactions can be used with primers derived from SEQ ID NO:1.

5 Expression of MraY

A variety of expression vectors can be used to express recombinant MraY in host cells. Expression vectors are defined herein as nucleic acid sequences that include regulatory sequences for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to express a bacterial gene in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Specifically designed vectors allow the shuttling of genes between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and regulatory sequences. Expression vectors can include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

In particular, a variety of bacterial expression vectors can be used to express recombinant MraY in bacterial cells. Commercially available bacterial expression vectors which are suitable for recombinant MraY expression include, but are not limited to pQE (Qiagen), pET11a (Novagen), lambda gt11 (Invitrogen), and pKK223-3 (Pharmacia).

Alternatively, one can express *mraY* DNA in cell-free transcription-translation systems, or *mraY* RNA in cell-free translation systems. Cell-free synthesis of MraY can be in batch or continuous formats known in the art.

One can also synthesize MraY chemically, although this method is not preferred.

A variety of host cells can be employed with expression vectors to synthesize MraY protein. These can include *E. coli*, *Bacillus*, and *Salmonella*. Insect and yeast cells can also be appropriate.

Following expression of MraY in a host cell, MraY polypeptides can be recovered. Several protein purification procedures are available and suitable for use. MraY protein and polypeptides can be purified from cell lysates and extracts, or from culture medium, by various combinations of, or individual application of methods including ultrafiltration, acid extraction, alcohol precipitation, salt

fractionation, ionic exchange chromatography, phosphocellulose chromatography, lecithin chromatography, affinity (*e.g.*, antibody or His-Ni) chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and chromatography based on hydrophobic or hydrophilic interactions. In some
5 instances, protein denaturation and refolding steps can be employed. High performance liquid chromatography (HPLC) and reversed phase HPLC can also be useful. Dialysis can be used to adjust the final buffer composition.

10 The following examples are presented in order to illustrate the instant invention.

EXAMPLE 1

General Materials and Methods

15 All reagents were purchased from SIGMA CHEMICAL CO., St. Louis, MO, unless otherwise indicated.

DNA manipulations reagents and techniques.

20 Restriction endonucleases and T4 ligase were obtained from Gibco-BRL. Agarose gel electrophoresis and plasmid DNA preparations were performed according to published procedures (Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989, Molecular cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory). Recombinant plasmids containing *P. aeruginosa* *mraY* were propagated in *E. coli* DH5 α (GIBCO-BRL, Rockville, MD) prior to
25 protein expression in *E. coli* BL21(DE3)/plysS (NOVAGEN, Madison, WI). SDS-PAGE was performed with precast gels (NOVAGEN). DNA sequences were determined using an automated ABI PRISMTM DNA sequencer (PERKIN-ELMER ABI, Foster City, CA).

EXAMPLE 2

Cloning of *Pseudomonas aeruginosa mraY*

Genomic DNA from *P. aeruginosa* (strain MB4439) was prepared from 100 ml late stationary phase culture in Brain Heart Infusion broth (DIFCO, Detroit, MI). Cells were washed with 0.2 M sodium acetate, suspended in 10 ml of TEG (100 mM Tris, pH 7, containing 10 mM EDTA and 25% glucose) and lysed by incubation with 200 µg of N-acetylmuramidase (SIGMA) for 1h at 37°C. Chromosomal DNA was purified from the cell lysate using a QIAGEN (Santa Clarita, CA) genomic DNA preparation kit following the manufacturers' protocol. Briefly, the cell lysate was treated with protease K at 50°C for 45 min, loaded onto an equilibrated QIAGEN genomic tip, and entered into the resin by centrifugation at 3000 rpm for 2 min. Following washing the genomic tip, the genomic DNA was eluted in distilled water and kept at 4°C. Approximately 50 ng genomic DNA was used as a template in PCR reactions to clone *mraY*.

Two oligonucleotide primers (GIBCO/BRL, Bethesda, MD) complementary to sequences at the 5' and the 3' ends of *P. aeruginosa mraY* were used to clone this gene using KLENTAQ ADVANTAGE™ polymerase (CLONTECH, Palo Alto, CA). The primer nucleotide sequences were as follows: 5'- TT CAT ATG CTC CTG CTG CTG GCC GAA TAC -3' (SEQ ID NO:3) and 5'- TT GGA TCC TCA ACG CAG CTT CAA GGT G -3' (SEQ ID NO:4). A PCR product representing *P. aeruginosa mraY* was verified by nucleotide sequence, digested with NdeI and BamHI, and cloned between the NdeI and BamHI sites of pET-11a, creating plasmid pPaeMraY. This plasmid was used for expression of the *mraY* gene in *E. coli*.

25

EXAMPLE 3

Overexpression and enzymatic activity of *Pseudomonas aeruginosa MraY*

mraY was cloned into the expression vector pET-11a (Novagen) as described above to create plasmid pPaeMraY. The pET-11a vector allows expression of authentic, non-fusion, proteins. The pET (Plasmids for Expression by T7 RNA polymerase) plasmids are derived from pBR322 and designed for protein over-

production in *E. coli*. The vector pET-11a contains the ampicillin resistance gene, and ColE1 origin of replication, in addition to T7 phage promoter and terminator. The T7 promoter is recognized by the phage T7 RNA polymerase but not by the *E. coli* RNA polymerase. A host *E. coli* strain such as BL21(DE3)pLysS is engineered 5 to contain integrated copies of T7 RNA polymerase under the control of lacUV5 that is inducible by IPTG. Production of a recombinant protein in the *E. coli* strain BL21(DE3)pLysS occurs after expression of T7RNA polymerase is induced.

The pPaeMraY plasmid was introduced into the host strain BL21 DE3/pLysS (NOVAGEN) for expression of MraY. Colonies were grown at 37°C in 10 100 ml of LB broth containing 100 mg/ml ampicillin and 32 µg/ml chloramphenicol. When cultures reached a cell density of $A_{600}=0.5$, cells were pelleted and then resuspended in M9ZB medium (NOVAGEN) containing 1 mM IPTG. Cells were induced for 3 h at 30°C, pelleted at 3000g, and frozen at -80°C.

Cultures containing either the recombinant plasmid pPaeMraY or the 15 control plasmid vector, pET-11a, were grown at 30°C and induced with IPTG. Lysates of cells transformed with pPaeMraY exhibited some 10.3 fold increase in MraY activity over uninduced cell lysates or induced lysates from cells containing the plasmid vector.

Assay for activity of MraY enzyme.

The MraY (translocase I) assay was performed using the butanol extraction method described by Brandish and coworkers (Brandish *et al.*, 1996 *J. Biol. Chem.* 271(13):7609-7614). The assay was performed at room temperature with assay components held at concentrations of: 100 mM TRIS, pH 7.5; 30 mM MgCl₂; 60.2 nCi [¹⁴C]UDP-MurNAc-pentapeptide (14 µM); 40 µM Decaprenol phosphate (Sigma Chemical Corp.); 0.15% Triton X-100 (w/v); and 100 mg/mL phosphatidyl glycerol (Sigma Chemical Corp.). Enzyme concentration was varied in order to obtain linear kinetics. Aliquots (50µl) were removed at varying time points and transferred to a fresh tube containing 50 µl of 6M pyridinium acetate, pH4.2. The mixture was then extracted with 100 µl butanol and 50 µl water. After brief centrifugation, 80 µl of the top butanol layer was quantitated in a Packard TriCarb scintillation counter to determine the amount of Lipid I product produced.

Table 1
Specific activity of recombinant MraY
from *P. aeruginosa*.

20

Recombinant MraY	Specific Activity	Fold Increase in Specific Activity
<i>P. aeruginosa mraY</i> in pET-11a vector	1428.2	10.3
Host <i>E. coli</i> cells containing pET-11a (empty vector – Control)	139.3	1

WHAT IS CLAIMED:

1. An isolated polynucleotide selected from the group consisting of:
 - 5 (a) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO:2,
 - (b) a polynucleotide which is complementary to the polynucleotide of (a), and
 - (c) a polynucleotide that hybridizes with a polynucleotide of (a) or 10 (b) under stringent conditions.
2. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1.
- 15 3. A polynucleotide that is an expression vector comprising a polynucleotide of claim 1.
4. A host cell comprising the expression vector of claim 3.
- 20 5. A process for expressing a MraY protein of *Pseudomonas aeruginosa* in a recombinant host cell, comprising:
 - (a) transforming a suitable host cell with an expression vector of claim 3; and
 - (b) culturing the host cell of step (a) in conditions under which 25 allow expression of the MraY protein from said expression vector.
6. cDNA selected from the group consisting of:
 - (a) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO:2,
 - (b) a polynucleotide which is complementary to the polynucleotide of (a), and
 - (c) a polynucleotide that hybridizes with a polynucleotide of (a) or 30 (b) under stringent conditions.

7. cDNA in accordance with claim 6 comprising the nucleotide sequence of SEQ ID NO:1.

8. An expression vector comprising the cDNA of claim 7.

5

9. A host cell comprising the expression vector of claim 8.

10. A process for expressing a MraY protein of *Pseudomonas aeruginosa* in a recombinant host cell, comprising:

10 11. (a) transforming a suitable host cell with an expression vector of claim 8; and

15 (b) culturing the host cell of step (a) in conditions under which allow expression of the MraY protein from said expression vector.

15 11. A probe for the detection of polynucleotides encoding *Pseudomonas aeruginosa* MraY protein, comprising at least 25 nucleotides of SEQ ID NO:1.

20 12. A probe in accordance with claim 11 selected from the group consisting of:

20 (a) a probe comprising a nucleotide sequence of SEQ ID NO:3;

and

(b) a probe comprising a nucleotide sequence of SEQ ID NO:4.

25 13. A primer for the detection of polynucleotides encoding *Pseudomonas aeruginosa* MraY protein, comprising at least 25 nucleotides of SEQ ID NO:1.

30 14. A primer in accordance with claim 13 selected from the group consisting of:

(a) a primer comprising a nucleotide sequence of SEQ ID NO:3;

and

(b) a primer comprising a nucleotide sequence of SEQ ID NO:4.

15. An isolated polypeptide comprising an amino acid sequence of SEQ ID NO:2.
16. A cellular extract comprising the polypeptide of claim 15.

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International Bureau



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(21) International Application Number: PCT/US00/27056

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(30) Priority Data:
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(71) Applicant (for all designated States except US): MERCK
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NJ 07065-0907 (US).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventors; and

(75) Inventors/Applicants (for US only): EL-SHERBEINI,



WO 01/25251 A1

(54) Title: MRAY GENE AND ENZYME OF *PSEUDOMONAS AERUGINOSA*

(57) Abstract: Polynucleotides and polypeptides of *Pseudomonas aeruginosa* MraY, an enzyme involved in bacterial cell wall biosynthesis, are provided. The recombinant MraY enzyme is catalytically active in the first step of the membrane cycle of peptidoglycan biosynthesis. Also provided are proteins encoded by the sequences, host cells expressing the recombinant enzyme, and probes and primers.

1/1

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FIG.1

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)		Attorney Docket Number	20522P
		First Named Inventor	El-Sherbeini, M. et al.
COMPLETE IF KNOWN			
		Application Number	
		Filing Date	
		Group Art Unit	
		Examiner Name	

Declaration Submitted with Initial Filing Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)
OR

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MraY GENE AND ENZYME OF PSEUDOMONAS AERUGINOSA

(Title of the Invention)

the specification of which

is attached hereto

OR

was filed on (MM/DD/YYYY) 09/29/2000 ✓ as United States Application Number or PCT International

Application Number PCT/US00/27056 ✓ and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Attorney Docket Number	Priority Claimed? YES NO
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Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	Attorney Docket Number
60/157,580 ✓	10/04/1999 ✓	20522PV

Please type a plus sign (+) inside this box

Approved for use through 9/30/2000 OMB 651-0032
SUBSTITUTE for PTO/SB/01 (12-97), DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY for Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known to me to be material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Application Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/US00/27056	09/29/2000 ✓	

Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint, respectively and individually, as my attorneys or agents with full power of substitution and revocation, the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Customer Number _____ → Place Customer Number Bar Code Label here
 Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
Anna L. Cocuzzo	42,452	Jack L. Tribble	32,633

2- Direct all correspondence to: Customer Number or Bar Code Label

000210

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		Fax	(732)594-4720

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:	<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])		Family Name or Surname						
Mohamed		El-Sherbeini						
Inventor's Signature	M El-Sherbeini			Date	2/27/02			
Residence: City	Westfield	NJ	State	NJ	Country	USA	Citizenship	US ✓
Post Office Address	Merck & Co., Inc., P.O. Box 2000							
City	Rahway	State	NJ	ZIP	07065-0907			
<input type="checkbox"/> Additional inventors are being named on the _____ supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.								

Please type a plus sign (+) inside this box



Approved for use through 9/30/2000 OMB 651-0032
SUBSTITUTE for PTO/SB/02A (3-97), Declaration (Additional Inventors)

DECLARATION AND POWER OF ATTORNEY

ADDITIONAL INVENTOR(S) Supplemental Sheet

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
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Inventor's Signature	<i>Barbara A. Azzolina</i>					Date	2/27/02 BA 2/27/02
Residence: City	Denville	NJ	State	NJ	Country	USA	Citizenship US <input checked="" type="checkbox"/>
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	State	NJ	ZIP	07065-0907		
Name of Additional Joint Inventor, if any:	<input type="checkbox"/> A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])		Family Name or Surname					
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
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Name of Additional Joint Inventor, if any:	<input type="checkbox"/> A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])		Family Name or Surname					
Inventor's Signature						Date	
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Post Office Address	Merck & Co., Inc., P.O. Box 2000						
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AERUGINOSA

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 Asn Thr Tyr Pro Ala Gln Val Phe Met Gly Asp Val Gly Ala Leu Ala
 260 265 270
 Leu Gly Ala Ala Leu Gly Thr Ile Ala Val Ile Val Arg Gln Glu Ile
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 Val Leu Phe Ile Met Gly Gly Val Phe Val Met Glu Thr Leu Ser Val
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 Met Ile Gln Val Ala Ser Phe Lys Leu Thr Gly Arg Arg Val Phe Arg
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 Met Ala Pro Ile His His His Phe Glu Leu Lys Gly Trp Pro Asp Pro
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20522P

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